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METHODS FOR *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Field of the Invention

This invention relates to methods for plant tissue culture and plant regeneration and in particular this invention relates to methods for transforming maize using *Agrobacterium*.

Background of the Invention

Agrobacterium-mediated transformation methods have been used principally in dicotyledonous plants. *Agrobacterium*-mediated transformation in dicotyledons facilitates the delivery of larger pieces of heterologous nucleic acid as compared with other transformation methods such as particle bombardment, electroporation, polyethylene glycol-mediated transformation methods, and the like. In addition, *Agrobacterium*-mediated transformation appears to result in relatively few gene rearrangements and more typically results in the integration of low numbers of gene copies into the plant chromosome.

Monocotyledons are not a natural host of *Agrobacterium.* Although *Agrobacterium*-mediated transformation has been reported for asparagus (Bytebier B., et al. *Proc. Natl. Acad. Sci. USA* 84:5354-5349, 1987) and for *Dioscore bublifera* (Schafer et al. *Nature* 327:529-532, 1987), it was generally believed that plants in the family *Gramineae* could not be transformed with *Agrobacterium* (Potrykus I. *Biotechnology* 8:535-543, 1990).

Grimsley et al. (*Nature* 325:177-179, 1987) reported that cDNA from maize streak virus could be delivered to maize plants by *Agrobacterium tumefaciens* and that the plants became infected with the virus. The research did not demonstrate that the cDNA reached the maize genome nor did it demonstrate stable integration of streak virus nucleic acid. Later studies demonstrated that *Agrobacterium* could be used to deliver a kanamycin-resistance gene and a GUS (β-glucuronidase) gene to shoot apices of maize after shoot apex injury (Gould J. et al. *Plant Physiol.*

95:426-434, 1991 and U.S. Patent No. 5,177,010 to Goldman et al.). In these studies plants generated from the tissue exposed to *Agrobacterium* contained both transformed cells and non-transformed cells suggesting that the method did not uniformly deliver nucleic acid to the maize tissue.

European Patent Application Publication Number 604 662 A1 to Hiei et al. discloses a method for transforming monocotyledons using *Agrobacterium*. In this method, plant tissues were obtained from the monocotyledon maize and the tissues were exposed to *Agrobacterium* during the tissue dedifferentiation process. Hiei et al. disclose a maize transformation protocol using maize calli. Saito et al. disclose a method for transforming monocotyledons using the scutellum of immature embryos (European Application 672 752 A1). Ishida et al. also disclose a method specific for transforming maize by exposing immature embryos to *A. tumefaciens* (*Nature Biotechnology*, 1996, 14:745-750). The methods were optimized for inbred A188 maize lines. Transformation frequencies ranged from 12% to 30% at their highest for immature embryos from A188 lines that were 1.0-1.2 mm in length. Maize lines derived from crosses of A188 had significantly lower transformation frequencies ranging from 0.4% to about 5.3%. The transformation frequencies using A188 and A188 crosses are summarized in Table 1. A188 is not generally considered a commercially useful line and Ishida et al. failed to obtain recovery of stable transformants in lines other than those containing A188.

A need still exists for a method that will: (a) produce significantly higher transformation frequencies in lines other than those reported by Ishida et al. (*supra*); and, (b) produce transformed inbred lines other than line A188; including transformed inbreds representing a range of genetic diversities and having significant commercial utility.

Summary of the Invention

This invention relates to methods for optimizing *Agrobacterium*-mediated transformation in maize. Significantly higher transformation frequencies for genotypes such as the product of A188 crossed to other inbreds would result in a higher throughput for production of transformed plants. This increased frequency would be useful, for example, to evaluate the efficacy of a larger number of genes in transgenic plants of corn or to generate a larger number of transgenic plants containing a particular foreign gene in a given period of time. Similarly, methods permitting the transformation of a variety of inbred lines would be commercially valuable.

In one aspect of this invention, the invention relates to a method for transforming maize using *Agrobacterium* comprising the steps of: contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to the embryo; co-cultivating the embryo with *Agrobacterium*; culturing the embryo in a medium comprising N6 salts, an antibiotic capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and regenerating maize plants expressing the gene. In one embodiment the contacting step additionally comprises the step of contacting the immature embryos with *Agrobacterium* in a medium comprising N6 salts and in another embodiment the contacting step additionally comprises contacting the immature embryos with *Agrobacterium* in a medium comprising MS salts. Preferably the contacting step takes place in the absence of $AgNO_3$. In one embodiment the embryos are cultured in a PHI basic media system and in another embodiment the embryos are cultured in a PHI combined media system. The immature embryos used in the method are preferably about 0.3 mm to about 4 mm in length and more preferably about 0.8 mm to about 2.0 mm in length. The *Agrobacterium* concentration used in the contacting step is preferably about 1×10^8 cfu/ml to about 1.5×10^9 cfu/ml and more preferably about 0.5×10^9 to about 1.0×10^9 cfu/ml. The contacting step preferably takes place in a liquid suspension and the

co-cultivation step preferably takes place on a solid medium. Preferably, a medium containing MS salts is used in the regeneration step. In a preferred embodiment of this invention the method includes a resting step that comprises culturing the embryos in medium containing an antibiotic capable of inhibiting the growth of *Agrobacterium*. Preferably the embryos are cultured for about 1 to about 15 days. In one embodiment the antibiotic used is carbenicillin and a preferred concentration of carbenicillin is about 50 mg/l to about 250 mg/l. This method also relates to maize plants transformed by this method and to maize cells transformed by this method.

In another aspect of this invention, the invention relates to a method for transforming maize using *Agrobacterium* comprising the steps of: contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to said embryo in a medium comprising N6 salts; co-cultivating the embryo with *Agrobacterium* in a medium comprising N6 salts; culturing the embryo in a medium comprising N6 salts, an antibiotic capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and regenerating plants expressing the gene in a medium comprising MS salts. Preferably, the medium of the contacting step lacks $AgNO_3$ and the medium of the co-cultivating step includes $AgNO_3$. Preferably the *Agrobacterium* concentration used in the contacting step is about 1×10^8 cfu/ml to about 1.5×10^9 cfu/ml. Preferably, the contacting step takes place in a liquid and the co-cultivating and culturing steps take place on a solid medium. In one embodiment of this method, the method additionally comprising the step of resting the embryo by culturing the embryo in a medium containing an antibiotic capable of inhibiting the growth of *Agrobacterium*. Preferably the antibiotic is carbenicillin. This invention also relates to maize plants and to maize cells transformed by this method.

In yet another aspect of this invention, a method is disclosed for transforming maize using *Agrobacterium* comprising the steps of: contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of

one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to said embryo in a medium comprising N6 or MS salts; co-cultivating the embryo with *Agrobacterium* in a medium comprising MS salts; culturing the embryo in a medium comprising N6 salts, an antibiotic capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and regenerating plants expressing the gene in a medium comprising MS salts. Preferably the medium of the contacting step lacks $AgNO_3$ and the method of the co-cultivating step includes $AgNO_3$. Also preferably, the contacting step takes place in a liquid and the co-cultivating and culturing steps take place on a solid medium. In one embodiment of this method, the method additionally comprising the step of resting the embryo by culturing the embryo in a medium containing an antibiotic capable of inhibiting the growth of *Agrobacterium*.

This invention also relates to a method for optimizing the production of transgenic maize plants of a first genotype using *Agrobacterium*-mediated transformation comprising the steps of: isolating immature embryos from maize; separating the embryos into treatment groups; incubating each treatment group separately in a medium comprising N6 or MS salts and in a suspension of *Agrobacterium* at concentrations ranging from about 1×10^8 cfu/ml to about 1×10^{10} cfu/ml; co-cultivating the embryos with *Agrobacterium* on a solid medium; culturing the embryos in a medium comprising N6 salts, an antibiotic capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos transformed by *Agrobacterium*; identifying the treatment group with the highest transformation frequency ; and using the concentration of *Agrobacterium* generating the highest transformation frequency to transform other embryos from the first genotype. In one embodiment of this method, the medium of the incubating step and the co-cultivating step is a medium comprising N6 salts and in another embodiment of this method, the medium of the incubating step is a medium comprising MS salts and the medium of the co-cultivating step is a medium

comprising N6 salts. In yet another embodiment, medium of the incubating step is a medium comprising N6 salts and the medium of the co-cultivating step is a medium comprising MS salts. The method also preferably includes the step of resting the embryo by culturing the embryo in a medium containing an antibiotic capable of inhibiting the growth of *Agrobacterium*. Preferably the antibiotic is carbenicillin and preferably, the combined length of the co-cultivating step and the resting step is at least three days. Where a resting step is used, the length of the resting step is from more than 0 to about 10 days. In a preferred embodiment, the length of the resting step is about 3 to about 5 days.

In another aspect of this invention, the invention relates to transformed maize plants produced by a method comprising the steps of: contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to the embryo; co-cultivating the embryo with *Agrobacterium*; culturing the embryo in a medium comprising N6 salts, an antibiotic capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and regenerating plants expressing the gene.

In yet another aspect of this invention, the invention relates to transformed maize cells produced by a method comprising the steps of: contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to the embryo; co-cultivating the embryo with *Agrobacterium*; and culturing the embryo in a medium comprising N6 salts, an antibiotic capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene: and identifying embryos expressing the gene.

In a preferred aspect of this invention, the invention relates to a method for transforming maize using *Agrobacterium* comprising the steps of: contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to the embryo; co-cultivating the embryo

with *Agrobacterium*; culturing the embryo in a medium containing salts other than MS salts, an antibiotic capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and regenerating plants expressing the gene.

Brief Description of the Figures

Fig.1 provides a diagram illustrating the construction of a preferred vector of this invention. Fig. 1(a) diagrams the exemplary gene segments incorporated into the exemplary vectors used in a preferred method of this invention. Fig. 1(b) illustrates plasmid pPHP8904 incorporating the exemplary gene segments. Fig. 1(c) illustrates plasmid pPHP10525.

Detailed Description of the Preferred Embodiments

The development of maize hybrids requires, in general, the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Pedigree breeding and recurrent selection breeding methods are used to develop inbred lines from breeding populations. Breeding programs combine the genetic backgrounds from two or more inbred lines or various other broad-based sources into breeding pools from which new inbreds are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with the inbred lines and the hybrids from these crosses are evaluated to determine which of those have commercial potential.

Pedigree breeding starts with the crossing of two genotypes, each of which may have one or more desirable characteristics that is lacking in the other or which complements the other. If the two original parents do not provide all of the desired characteristics, other sources can be included in the breeding population. A single cross hybrid maize variety is the cross of two inbred lines, each of which has a genotype that complements the genotype of the other. The hybrid progeny of the first generation is designated F_1. In the development of hybrids, only the F_1 hybrid

plants are sought. Preferred hybrids are more vigorous than their inbred parents. This hybrid vigor, or heterosis, can be maintained in many polygenic traits, including increased vegetative growth and increased yield.

The development of a hybrid maize variety involves three steps: (1) selection of plants from various germplasm pools for initial breeding crosses; (2) the selfing of the selected plants from the breeding crosses for several generations to produce a series of inbred lines, that, although different from each other, are highly uniform; and (3) crossing the selected inbred lines with different inbred lines to produce the hybrid progeny (F_1). Inbred lines produced in such a breeding program naturally fall into what are termed different heterotic groups. Maximal heterosis, or hybrid vigor, is typically produced by crossing two inbreds, each from a different heterotic group. At least several distinct heterotic groups can be identified, with numerous inbreds belonging to each heteotic group. A significant amount of research and development goes into the identification and recovery of inbred lines of commercial importance. For example, some 400-500 new inbred lines may be proposed by a single seed corn corporation each year as a result of over 2,000,000 pollinations. Of those proposed new inbreds, fewer than 50 and more commonly fewer than 30 are selected for commercial use. Those inbred lines that are used in commercially important hybrids are considered to be "elite" inbred lines. Not only is there a need to directly transform inbred lines that are commercially important for the hybrid corn market but there is also a need for those inbreds to cover a wide range of genetic diversity.

A188 is a useful genotype for the development of corn transformation methods, since it is known to be highly responsive in producing a friable type of embryogenic callus that lends itself to tissue culture (Phillips, R. L. et al. "Cell/Tissue Culture and In Vitro Manipulation" pp. 345-387, in *Corn and Corn Improvement*, G.F. Spraque and J.W. Dudley, eds., American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc. 1988 and Armstrong, C.L. et al., *Maize Genet. Coop. News Letter* 59:92-93, 1985).

However, A188 is no longer generally considered to be a useful inbred parent of commercial hybrid corn. A188 is not used directly in any commercial hybrid and is a poor starting material for backcrossing into inbreds used as parents of commercial hybrids. Use of non-elite starting material for back crossing will in some cases delay release of the final commercial hybrid by one to two years. In addition, with non-elite starting material, there is a higher risk of negative genetic effects on the final hybrid product. Therefore, the ability to transform only A188-containing lines is of limited value to the commercial hybrid corn market.

Although Hiei et al. and Ishida et al., both *supra*, were successful in using *Agrobacterium* to transform A188-containing maize lines, non-A188 inbred lines could not be transformed using this method (Ishida et al. *Nature Biotechnology* 14:745-750, 1996). Some of the non-A188 inbreds tested included lines that were useful for hybrid field corn breeding. A significant need still exists for methods to transform non-A188 inbred lines, including inbred lines that are commercially important for the hybrid corn market. The commercial corn market includes a wide range of hybrids with different genetic backgrounds and successful inbred breeding programs need to cover a wide range of genetic diversity.

High efficiency transformation of maize is important in analyzing the usefulness of any of a variety of genes in transgenic corn plants. High efficiency transformation of maize is also important because large numbers of transgenic plants are needed to study the effect of a particular gene within a given period of time. The ability to directly transform agronomically important inbreds at a usable frequency and across a wide range of genetic diversity is important for the development of commercial hybrid seed products with improved traits including, but not limited to, insect resistance, disease resistance, herbicide resistance, increased yield, increased tolerance to environmental stresses (such as drought, heat, etc.), enhanced seed quality (such as increased or modified starch, oil and/or protein content), and the like.

Although non-*Agrobacterium*-mediated transformation methods are known, including, but not limited to, particle bombardment, electroporation and silicon carbide fiber-mediated transformation (Songstad, D.D., et al. *Plant Cell, Tissue and Organ Culture* 40:1-15, 1995), the utility of these methods is limited because the methods produce low transformation frequencies and/or because the methods may only be useful for a restricted number of genotypes. For example, transformation frequencies by bombardment have been reported to be less than 2% for the "Hi-II" genotype used in the present invention (Songstad, D.D. et al. *In Vitro Cell. Dev. Biol. Plant* 32:179-183, 1996), even though Hi-II is one of the more responsive and efficient maize genotypes in tissue culture. Protoplast systems have also been used for transformation studies including electroporation and polyethylene glycol (PEG)-mediated methods for nucleic acid uptake. Reports indicate that maize protoplast systems can show good transformation frequencies (Donn, G., in *Abstracts of the VIIth International Congress on Plant Cell and Tissue Culture*, IAPTC, A2-38, p. 53, 1990). The protoplast genotype used in this study was a specially derived complex synthetic maize genotype, He/89, that demonstrated good regeneration capability and had low rate of plant abnormalities (Morocz, S., et al. *Theoretical and Applied Genetics* 80:721-726, 1990). However, aside from the specialty lines such as HE89, most genotypes, including agronomically important genotypes are not very amenable to the use of protoplast targeted transformation methods. Consequently, Wilson et al. (in *Transformation of Plants and Soil Microorganisms*, Wang, Herrera-Estrella et al. eds. Cambridge University Press, p. 65-80, 1995) conclude that "genotype constraints and the reduced vigor and fertility of plants regenerated from protoplasts probably outweigh the benefits of protoplasts as recipients for the integration of foreign DNA." Only a limited number of genotypes are amenable to the use of protoplasts for transformation and the quality of the plant produced from protoplast culture is often not as good as the quality of the plant produced from other transformation systems.

Ishida et al. (*supra*) discuss a method for transforming inbred A188 embryos and F_1 embryos derived from crosses of A188 with other inbred lines through the co-cultivation of the embryos with *Agrobacterium tumefaciens* using superbinary vectors. Table 1 of that publication summarized the frequencies for maize transformation obtained in that study. Maximum transformation frequencies reached 30.6% for A188 but the transformation frequency only reached 5.3% for embryos derived from crosses of A188 and the average transformation frequency for A188 was about 15%. Non-A188-containing lines could not be transformed by these methods. The transformation frequencies using embryos derived from A188 and F_1 embryos from crosses of A188, as reported in Table 1 of the Ishida et al paper, are summarized in Table 1 below. The transformation frequencies of F_1 embryos ranged from 0.4-5.3% and was defined as the proportion of total embryos which produced GUS expressing (GUS+) plants. In this table, and as used herein, the term "GUS+" refers to transgenic events where GUS gene expression can be detected.

Table 1: Transformation of A188 and crosses by Ishida et al.

The results in this Table were reported in Ishida et al., (*Nature Biotechnology* 14:745-750, 1996). GUS+ plants are those which showed positive staining for expression of the GUS gene.

		Number of immature embryos				
Variety	Experiment No.	Inoculated (A)	Callus growing on herbicide	Plants regenerated on herbicide	GUS+ plants (B)	Frequency B/A, %)
A188	1	44	28	9	6	13.6
	2	52	33	10	7	13.5
	3	51	46	13	7	13.7
	4	70	56	26	14	20.0
	5	76	30	12	9	11.8
	6	369	200	71	44	11.9
	7	121	46	33	20	16.5
	8	27	15	8	5	18.5
	9	36	26	18	11	30.6
	10	77	38	32	16	20.8
W117xA188	1	112	36	8	4	3.6
	2	114	26	10	6	5.3
W59ExA188	1	104	44	1	1	1.0
A554xA188	1	247	46	7	5	2.0
W153RxA188	1	284	69	2	1	0.4
H99xA188	1	219	18	4	3	1.4

The present invention, while complementing the work of Ishida et al., provides an improved method for generating a significant increase in *Agrobacterium*-mediated transformation frequency for A188-containing lines and for successfully transforming non-A188 inbreds across a wide range of genotypes. In this invention, methods described by Ishida et al. (including the same source of A188 and the same vector) were used to transform A188 lines to demonstrate that the Ishida et al. methods could be reproduced in a different laboratory, on the same A188 genotype. The results of these initial transformation studies are provided in Example 2 and the data are summarized in Table 3. The results of these experiments produced transformation frequencies similar to those reported by Ishida et al. and ranged from about 9% to about 18% for A188 transformation in four separate experiments.

The methods of Ishida et al. were then used to transform a genotype termed Hi-II. This provided a baseline for transformation frequencies that could be used as a comparison with the transformation protocols of this invention. Hi-II is similar to the A188 x inbred crosses used by Ishida et al. (i.e., those listed in Table 1) to the extent that Hi-II is derived from both A188 and a non-A188 inbred, B73 (Armstrong et al. *Maize Genetics Cooperation Newsletter* 65:92-93, 1991). Details on the derivation of Hi-II and the results of the Hi-II transformation studies using the Ishida et al. method, are provided in Example 3. The data are summarized in Table 4. The results of these experiments show that transformation frequencies obtained for Hi-II, using the protocols of Ishida et al., ranged from 0.8 to 7.1% and were also in the same general range of transformation frequencies as those obtained by Ishida et al. for A188 inbred crosses. The results reported in Example 2 and Example 3 demonstrated that the method of Ishida et al. was reproducible by others. These results allow comparisons to be made between the new *Agrobacterium*-mediated transformation methods of this invention and those reported in the literature.

The preferred *Agrobacterium*-mediated transformation process of this invention differs from that of Ishida et al. and Hiei et al. in several respects and can be broken into several steps.

As will be discussed in more detail below, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium* (step 1; the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2; the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4; the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5; the regeneration step) and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

Infection Step

As a first step for practicing this invention, immature embryos are isolated from maize and exposed to *Agrobacterium*. Immature embryos are an intact tissue that is capable of cell division to give rise to callus cells that can then differentiate to produce tissues and organs of a whole plant. Immature embryos can be obtained from the fertilized reproductive organs of a mature maize plant.

Exemplary methods for isolating immature embryos from maize are described by Green and Phillips (*Crop Sci.* 15:417-421, 1976). Maize immature embryos can be isolated from pollinated plants, as another example, using the methods of Neuffer et al. ("Growing Maize for genetic purposes." In : *Maize for Biological Research* W.F. Sheridan, Ed.,University Press, University of North Dakota, Grand Forks, North Dakota. 1982.). Another method is provided in Example 4. The immature embryos are preferably used at approximately 6 days to about 20 days after pollination, more preferably about 7 days to 18 days after pollination, still more preferably about 8 days to 16 days after pollination, and in a particularly preferred embodiment about 9 days to about 12 days after pollination. Preferably, the embryos exposed to *Agrobacterium* range from about 0.3 to 4 mm in size, more preferably about 0.6 to 3.0 mm, still more preferably about 0.8 to 2.0 mm and in a particularly preferred embodiment about 1.0 mm to about 1.2 mm in size. Immature embryos are preferably aseptically isolated from the developing ear and held in sterile medium until use.

The *Agrobacterium* used to transform the embryos is modified to contain a gene of interest. Preferably the gene is incorporated into a gene vector, to be delivered to the embryo. A variety of *Agrobacterium* species are known and *Agrobacterium* species employed for dicotyledon transformation can be used. A number of references review *Agrobacterium*-mediated transformation in monocots and dicots. These include, among others, Hooykaas, P.J. (*Plant Mol. Biol.*, 13:327-336, 1989); Smith, R.H. et al. (*Crop Science*, 35:301-309, 1995); Chilton, M.O. (*Proc. Natl. Acad. Sci.* (USA), 90:3119-3210, 1993); and Moloney et al. In: *Monograph Theor. Appl. Genet.*, NY, Springer Verlag 19:148-167, 1993).

Many *Agrobacterium* employed for the transformation of dicotyledonous plant cells contain a vector having a DNA region originating from the virulence (*vir)* region of the Ti plasmid. The Ti plasmid originated from *Agrobacterium tumefaciens*. Nucleic acid containing a gene encoding a polypeptide to be expressed in maize can be inserted into this vector. Alternatively, the gene can

be contained in a separate plasmid which is then inserted into the Ti plasmid *in vivo*, in *Agrobacterium*, by homologous recombination or other equivalently resulting processes. A vector has also been developed which contains a DNA region originating from the virulence (*vir*) region of Ti plasmid pTiBo542 (Jin et al., 1987, *J. Bacteriol.* 169:4417-4425) contained in a super-virulent *Agrobacterium tumefaciens* strain A281 showing extremely high transformation efficiency. The plasmid containing the gene of interest was incorporated into the virulent *Agrobacterium tumefaciens* strain A281 since strain A281 is known to have a high transformation efficiency (see Hood, E.E. et al.,1984, *Bio/Tech* 2:702-709; Komari, T. et al., 1986, *Bacteriol* 166:88-94). This type of vector is known in the art as a "superbinary vector" (see European Patent Application 0 604662A1 to Hiei et al.).

Superbinary vectors are preferred vectors for the transformation methods of this invention. Exemplary superbinary vectors useful for introducing nucleic acid encoding polypeptide for expression in a maize plant via *Agrobacterium*-mediated transformation methods include the superbinary pTOK162 (as disclosed in Japanese Laid-Open Patent Application no. 4-222527). This vector includes regions that permit vector replication in both *E. coli* and *A. tumefaciens*. The plasmid includes a T-DNA region, characteristic of Ti plasmids. Nucleic acid containing a gene encoding a polypeptide to be expressed in maize is inserted in the T-DNA between the T-DNA borders. Other superbinary vectors are known and these vectors can similarly be incorporated into *Agrobacterium* (see e.g., Komari, T., *Plant Cell Reports* 9:303-306, 1990 for pTOK23).

Examples of genes useful for expression in transformed plant cells are known in the art. Exemplary genes include, but are not limited to, Bt genes or patatin genes for insect resistance; the Hm1 gene and chitinase genes for disease resistance; the pat, bar, EPSP syntase gene or ALS genes for herbicide resistance; genes encoding proteins with altered nutritional properties; genes encoding enzymes involved in starch or oil biosynthetic pathways; down-or up-regulatory sequences for metabolic pathway enzymes; and the like. As those of ordinary skill in the art

will recognize, this is only a partial list of possible genes that can be used with the transformation method of the present invention. Furthermore, as those of ordinary skill in the art will also recognize, regulatory sequences including promoters, terminators and the like will also be required, and these are generally known in the art. Example 1 discloses the construction of a preferred superbinary vector pPHP10525. This vector contains virB, virC and virG genes isolated from superviral strain A281. The vector includes 35Sbar and ubi/GUS plant expression cassettes inserted between the T-DNA borders. Plant expression cassettes preferably comprise a structural gene to which is attached regulatory DNA regions that permit expression of the gene in plant cells. The regulatory regions consist at a minimum of a promoter capable of directing expression of a gene in a plant cell. The promoter is positioned upstream or at the 5' end of the gene to be expressed. A terminator is also provided as a regulatory region in the plant expression cassette and is capable of providing polyadenylation and transcription terminator functions in plant cells. The terminator is attached downstream or at the 3' end of the gene to be expressed. Marker genes, included in the vector, are useful for assessing transformation frequencies in this invention.

The nucleic acid encoding a polypeptide for expression in maize is inserted into the T-DNA region of the superbinary vector using suitable restriction endonuclease recognition sites, by homologous recombination, or the like. General molecular biological techniques used in this invention are provided, for example, by Sambrook, et al. (eds.) (*Molecular Cloning: A Laboratory Manual*, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and the use of homologous recombination to incorporate nucleic acid into plasmids contained in *Agrobacterium tumefaciens* is disclosed by Herrera-Esterella, L. et al. *(EMBO J.* 2:987-995, 1983) and Horsch R.H. et al., (*Science* 223:496-498, 1984). The recombinant plasmid is selected in *Agrobacterium* based on the use of a selectable marker incorporated into the plasmid. Generally these markers are nucleic acid encoding proteins that typically confer antibiotic resistance.

Plasmids are introduced into *Agrobacterium* using methods known in the art, including the triple-cross method disclosed by Ishida et al. (*supra)* and in a preferred embodiment the plasmid is introduced into *Agrobacterium* using the method of Example 1.

Agrobacterium containing the plasmid of interest are preferably maintained on *Agrobacterium* master plates with stock frozen at about -80°C. As used in this invention the term "*Agrobacterium* capable of transferring at least one gene" refers to *Agrobacterium* containing the gene of interest, generally in a plasmid that is suitable for mediating the events required to transfer the gene to the cells to be infected. In a preferred embodiment, the master plates are used to inoculate agar plates to obtain *Agrobacterium* which is then resuspended in media for use in the infection process as described in Example 2. Alternatively, bacteria from the master plate can be used to inoculate broth cultures that are grown to logarithimic phase prior to transformation.

The concentration of *Agrobacterium* used in the infection step and co-cultivation step can affect the transformation frequency. For example, while *Agrobacterium* can transform immature embryos of maize, very high concentrations of *Agrobacterium* may also damage the immature embryos and result in a reduced callus response. To optimize the transformation protocol for a particular maize line, immature embryos from the maize line can be incubated with various concentrations of *Agrobacterium*. Using the protocols provided in Examples 2-6, the level of marker gene expression and the transformation efficiency can be assessed for various *Agrobacterium* concentrations preferably within the concentration range of about 1.0 x 10^8cfu/ml to about 1 x 10^{10} cfu/ml. Table 6 in Example 4 demonstrated the effect of varying *Agrobacterium* concentration to optimize the *Agrobacterium* concentration for transformation. Using these methods, and those known in the art, concentrations of *Agrobacterium* in the infection and co-cultivation step that maximize the transformation frequency for a particular maize line can be identified without undue experimentation.

Preferably, *Agrobacterium* is used for transformations in a concentration range of about 1 x 10^8 cfu/ml to about 1 x 10^{10} cfu/ml, more preferably within the range of about 1 x 10^8 cfu/ml to about 1.5 x 10^9 cfu/ml and still more preferably at about 0.5 x 10^9 cfu/ml to about 1.0 x 10^9 cfu/ml. Those skilled in the art will recognize that optimum *Agrobacterium* concentration ranges may vary for particular maize genotypes and for the particular *Agrobacterium* strain.

The isolated embryos are added to the *Agrobacterium* suspension in a liquid contact phase. Preferably the *Agrobacterium* concentration is selected based on methods disclosed herein to optimize transformation efficiencies. Example 4 provides a preferred method for contacting the embryos with *Agrobacterium* in a liquid. The contact phase facilitates maximum contact of the immature embryos with the suspension of *Agrobacterium*. Preferably the embryos are contacted with the suspension of *Agrobacterium* for a period of at least 5 minutes and preferably between 5 to 15 minutes and more preferably for about 10 minutes.

Preferably the liquid contact phase of the infection step takes place in a liquid solution that includes the major inorganic salts and vitamins of N6 medium referred to herein as "N6 salts" (Chu C.C. *Proc. Symp. Plant Tissue Culture*, Science Press Peking. pp.43-50, 1987). As used herein, medium containing "N6 salts" includes medium containing about 400-500 mg/l ammonium sulfate and preferably about 463.0 mg/l ammonium sulfate; about 1.0-2.0 mg/l boric acid and preferably about 1.6 mg/l boric acid; about 100-140 mg/l calcium chloride anhydrous and preferably about 125 mg/l calcium chloride anhydrous; about 20-50 mg/l Na_2-EDTA and preferably about 37.25 mg/l Na_2-EDTA; about 20-40 mg/l ferrous sulfate.$7H_2O$ and preferably about 27.8 mg/l ferrous sulfate.$7H_2O$; about 80-100 mg/l magnesium sulfate and preferably about 90.37 mg/l magnesium sulfate.H_2O, about 1.5-7 mg/l manganese sulfate.H_2O and preferably about 3.33 mg/l manganese sulfate; about 0.4-1.6 mg/l potassium iodide and preferably about 0.8 mg/l potassium iodide; about 1,500-3,500 mg/l potassium nitrate and preferably about 2,830 mg/l potassium nitrate; about 200-600 mg/l potassium phosphate

monobasic and preferably about 400 mg/l potassium phosphate monobasic; and, about 1.0-2.5 mg/l zinc sulfate.$7H_2O$ and preferably about 1.25-1.75 mg/l zinc sulfate.$7H_2O$. Other equivalent liquid suspensions can be used and, as summarized in Table 2, media containing MS salts was also successfully used in the infection step. MS salts include about 1,650.0 mg/l ammonium nitrate, about 6.2 mg/l boric acid, about 332.2 mg/l calcium chloride anhydrous, about 0.025 mg/l cobalt chloride.$6H_20$, about 0.025 mg/l cupric sulfate.$5H_2O$, about 37.26 mg/l Na_2-EDTA, about 27.8 mg/l ferrous sulfate.$7H_2O$, about 180.7 mg/l magnesium sulfate.H_2O, about 16.9 mg/l manganese sulfate.H_2O, about 0.83 mg/l potassium iodide, about 1,900.0 mg/l potassium nitrate, about 170.0 mg/l potassium phosphate monobasic, and about 8.6 mg/l zinc sulfate.$7H_2O$. Three different media, PHI-A, PHI-G and PHI-I, were tested in the infection step and these formulations are provided in the Examples.

Preferred media used in this step is provided in Example 4. In addition, the media in the infection step preferably excludes $AgNO_3$. $AgNO_3$ is preferably included in the co-cultivation, resting (when used) and selection steps when N6 media is used.

Those skilled in the art will recognize that although this method is disclosed for embryos isolated from maize, the method can also be used to transform maize cell suspensions. Therefore, the term "plant cells" as used in this invention can refer to isolated maize cells, including suspension cultures as will as to cells in an intact tissue, such as maize embryos.

Co-cultivation Step

In a next step of a preferred transformation protocol of this invention, the immature embryos are co-cultivated with the *Agrobacterium* on a solid medium. The embryos are preferably positioned axis down on the solid medium and the medium preferably includes $AgNO_3$ at a range of about 0.85 to 8.5 mg/l, although 0.01 to 200 mg/l can also be used. The embryos are preferably cocultivated with the

Agrobacterium for about 1-30 days, preferably about 2-20 days and more preferably about 3-10 days.

Two media regimes have been identified as useful in the methods of this invention: PHI basic medium and PHI combined medium. A summary of the media regimes used is provided in Table 2. The PHI basic medium contains N6 salts and is used in one embodiment of this invention, in the infection, co-cultivation optional resting and selection steps of this invention; MS salts are preferably used in the regeneration step. The PHI combined medium contains either N6 or MS salts in the infection step, MS salts in the co-cultivation step, N6 salts in the optional resting step and in the selection step and preferably MS salts in the plant regeneration step (Table 2). As illustrated in Examples 4-6, both basic media, containing N6 salts (for example, PHI-B), and the combined medium, using MS salts (for example, PHI-J), in the co-cultivation step demonstrated improved transformation efficiencies.

Preferably, where embryos are incubated on solid media containing N6 salts, the embryos remain on media containing N6 salts through the selection step. For embryos incubated in the co-cultivation step on MS containing medium, the embryos are preferably incubated in N6 salt-containing medium for the optional resting and the selection step. The preferred media combinations of this invention are summarized in Table 2.

Although Saito et al. and Hiei et al. cite the use of N6 salts for rice, Saito et al. specifically cite the use of LS salts rather than N6 salts in the examples for maize. Ishida et al. tested both LS and N6 salts for maize, but failed to obtain any stable transformants with N6 salts. Ishida et al. considered LS salts to be "superior to N6-based media" (*supra*) and used LS salts for every step of the process in all further experiments. Therefore, whenever the protocol of Ishida et al. is cited in the present application, it is understood that LS salts are included in that protocol. The macro and micro salts in MS medium are identical to the macro and micro salts in LS medium, but the two media differ in the composition of some of the vitamins

and other components (Skirvin R.M., In: *Cloning Agricultural Plants Via In Vitro Techniques*, B.V. Conger, ed., CRC Press, Knoxville, Tenn., pp. 51-140, 1981).

Optional Resting Step

Following the co-cultivation step, the embryos are optionally transferred to a second plate of solid medium containing an antibiotic capable of inhibiting the growth of *Agrobacterium*. This resting phase is performed in the absence of any selective pressures to permit preferential initiation and growth of callus from embryos containing the heterologous nucleic acid. Preferably, the antibiotic used to inhibit *Agrobacterium* is carbenicillin and the preferred concentrations of carbenicillin are about 50 mg/l to about 250 mg/l carbenicillin in the solid media, more preferably about 100-125 mg/l carbenicillin. A particularly preferred concentration of carbenicillin is about 100 mg/l. Other antibiotics can be used that inhibit the growth of *Agrobacterium* and these include for example Cefotaxime, timetin, vancomycin, and the like. Those of ordinary skill in the art of monocot transformation will recognize that the concentration of antibiotic can be optimized for a particular transformation protocol without undue experimentation. The resting phase cultures are preferably allowed to rest in the dark at 28°C for about 3 to about 5 days, but about 1 to about 15 days can also be used. Example 4 uses a 3-5 day resting period. A preferred resting step medium is PHI-C as provided in the examples. In addition, although Hiei et al. and Saito et al. describe use of a "resting" step for rice, no such resting step is cited for maize nor used in the Ishida et al. protocol for maize.

Example 5 provides a comparison of the surprising benefits achieved using a resting step for maize line Hi-II. In a preferred embodiment of this invention, a resting step is provided; however, a resting step is not always required and where no resting step is used, an extended co-cultivation step may be added to provide a period of culture time prior to the addition of a selective agent (see Example 7).

Selection Step

Following the co-cultivation step, or following the resting step, where it is used, the embryos are exposed to selective pressure to select for those cells that have received and are expressing polypeptide from the heterologous nucleic acid introduced by *Agrobacterium*. In the selection step, the embryos are transferred to plates with solid medium that includes both an antibiotic to inhibit growth of the *Agrobacterium* and a selective agent. The agent used to select for transformants will select for preferential growth of explants containing at least one selectable marker insert positioned within the superbinary vector and delivered by the *Agrobacterium*. Example 1 incorporates the bar gene into a superbinary vector that is introduced into the *Agrobacterium*. The bar gene confers herbicide resistance to glufosinate-type herbicides, such as phosphinothricin (PPT) or bialaphos, and the like. Bialaphos was used to select for embryos that received and expressed the bar gene in Examples 2-6. Examples of other selective markers that could be used in the vector constructs include, but are not limited to, the pat gene, also for bialaphos and phosphinothricin resistance, the ALS gene for imidazolinone resistance, the HPH or HYG gene for hygromycin resistance, the EPSP synthase gene for glyphosate resistance, the Hm1 gene for resistance to the Hc-toxin, and other selective agents used routinely and known to one of ordinary skill in the art.

Preferably, media containing salts other than MS salts is used in the selection step and in a preferred embodiment, media containing N6 salts is used in the selection step. Exemplary medias used in the selection step include PHI-D and PHI-H, as provided in the examples. During selection, the embryos are cultured until callus formation is observed. Typically, calli grown on selection medium are allowed to grow to a size of about 1.5 to 2 cm. diameter.

Plant Regeneration Step

After the calli have reached the appropriate size, the calli are cultured on regeneration medium in the dark for about 1 to 3 weeks to allow the somatic embryos to mature. Preferred regeneration media included media containing MS

salts, such as PHI-E and PHI-F media as provided in the Examples. The calli are then cultured on rooting medium in a light/dark cycle until shoots and roots develop. Methods for plant regeneration are known in the art and preferred methods are provided by Kamo et al. (1985, *Bot. Gaz.* 146(3):327-334), West et al. (1993, *The Plant Cell* 5:1361-1369), and Duncan et al. (1985, *Planta*, 165:322-332).

Small plantlets are then transferred to tubes containing rooting medium and allowed to grow and develop more roots for approximately another week. The plants are then transplanted to soil mixture in pots in the greenhouse.

The following table (Table 2) summarizes the preferred protocols of this invention.

Table 2. Summary of the steps, salts and antibiotic in PHI protocols for *Agrobacterium*-mediated maize transformation.

	Transformation steps				
Protocol	Step 1	Step 2	Step 3	Step 4	Step 5
	Infection	Co-cultivation	Resting	Selection	Regeneration
Ishida et al.	LS	LS	None disclosed	LS cefotaxime	LS cefotaxime
PHI Basic	N6	N6	N6 carbenicillin	N6 carbenicillin	MS carbenicillin
PHI combined	MS or N6	MS	N6 carbenicillin	N6 carbenicillin	MS carbenicillin

The methods of this invention were applied to the maize line Hi-II. These results are provided in Example 4 and the data summarized in Table 5. The results of these experiments demonstrated that the methods of this invention do provide an increased transformation frequency for Hi-II as compared with the Ishida et al. protocol (Example 3 and Table 4). Plant regeneration frequency from stably-transformed callus in Hi-II and in other genotypes ranged from about 95% to about 100%, demonstrating that either GUS+ callus or GUS+ plants can be used as an

indicator of transformation frequency. In Tables 4-8, GUS+ events represent either GUS+ callus or GUS+ plants. Therefore, in Table 4 and all subsequent tables, transformation frequency was calculated by dividing the number of embryos producing GUS+ events (either GUS+ callus or GUS+ plants) by the total number of embryos inoculated with *Agrobacterium*.

The methods of this invention were next applied to F_1 embryos of crosses between A188 and other inbred lines. The results of these transformation studies are provided in Example 6 and the data are summarized in Table 8. These results indicate that the methods of this invention produced an improved transformation frequency for crosses of A188 to the inbreds with transformation frequencies ranging from 6.9% to 47.7%. These transformation frequencies were significantly higher than the frequencies reported by Ishida et al. for A188 x inbred crosses which ranged from 0.4 to 5.3%. Taking the results in Table 4 (using the Ishida et al. method) together with the results in Table 8, the methods of this invention provide for significantly improved transformation frequencies for A188-containing lines.

The present invention also provides an improved method for the transformation of a variety of inbred lines other than A188, and importantly including maize lines across a wide range of genetic diversity. Three different elite inbred lines were tested, belonging to three different heterotic groups and therefore representing a broad range of genetic diversity. The method of Ishida et al. was also tested on these same three inbreds, for comparison, using 594, 644 and 263 embryos for lines PHJ90, PHN46 and PHP~~28~~38, respectively. The results are provided in Example 7 and the data summarized in Table 9. These experiments demonstrated that the methods of this invention are successful in transforming inbred lines covering a wide genetic range. In contrast, no stable transformants were recovered in any of the three inbred lines using the methods of Ishida, et al. (*supra*) or those of Hiei et al. (European Patent Application Publication Number 604 662 A1). These

results are significant because this data indicates that a variety of maize lines can be efficiently and consistently transformed.

The methods of this invention proved useful for a variety of maize lines. The data indicated that the methods of this invention provide substantial improvements in *Agrobacterium*-mediated transformation frequencies as compared to methods currently available in the art.

All references and publications cited herein are expressly incorporated by reference into this disclosure. Particular embodiments of this invention will be discussed in detail and reference has been made to possible variations within the scope of this invention. There are a variety of alternative techniques and procedures available to those of skill in the art which would similarly permit one to successfully perform the intended invention that do not detract from the spirit and scope of this invention.

Example 1
Construction of *Agrobacterium* vectors and strains

All vectors were constructed using standard molecular biology techniques (Sambrook et al., (eds.), *Supra*). A reporter gene and a selectable marker gene for gene expression and selection was inserted between the T-DNA borders of a superbinary vector. The reporter gene included the β-glucuronidase (GUS) gene (Jefferson, R.A. et al., 1986, *Proc. Natl. Acad. Sci. (USA)* 83:8447-8451) into whose coding region was inserted the second intron from the potato ST-LS1 gene (Vancanneyt et al., *Mol. Gen. Genet.* 220:245-250, 1990), to produce intron-GUS, in order to prevent expression of the gene in *Agrobacterium* (see Ohta, S. et al., 1990, *Plant Cell Physiol.* 31(6):805-813). Referring to Fig. 1(a), the 2 kb fragment of the promoter region of the maize ubiquitin gene Ubi-1 (Christensen et al., *Plant Mol. Biol.* 18:675-689, 1992), with added 5' HindIII and 3' BamHI restriction sites, was ligated to the 5' BamHI site of the GUS gene. A fragment containing bases 2 to 310 from the terminator of the potato proteinase inhibitor

(pinII) gene (An et al., *Plant Cell* 1:115-122, 1989) was blunt-end ligated downstream of the GUS coding sequence, to create the GUS expression cassette. The 3' end of the terminator carried a NotI restriction site.

For the selectable marker, a Cauliflower Mosaic Virus 35S promoter with a duplicated enhancer region (2X35S; bases -421 to -90 and -421 to +2 from Gardner et al., *Nucl. Acids Res.* 9:2871-2888, 1981) with a flanking 5' NotI site and a 3' PstI site was created. A PstI/SalI fragment containing the 79 bp Tobacco Mosaic Virus leader (Gallie et al., *Nucl. Acids Res.* 15:3257-3273, 1987) was inserted downstream of the promoter followed by a SalI/BamHI fragment containing the first intron of the maize alcohol dehydrognease gene ADH1-S (Dennis et al., *Nucl. Acids Res.* 12:3983-3990, 1984). The BAR coding sequence (Thompson et al., *EMBO J.* 6:2519-2523, 1987) was cloned into the BamHI site, with the pinII terminator ligated downstream, to create the BAR expression cassette. The pinII terminator was flanked by a 3' SacI site.

The plasmid, pPHP8904, (Fig.1b) was constructed by inserting the GUS expression cassette as a HindIII/NotI fragment and the BAR expression cassette as a NotI/SacI fragment between the right and left T-DNA borders in pSB11 at HindIII and SacI sites. The GUS cassette is inserted proximal to the right T-DNA border. The plasmid pSB11 was obtained from Japan Tobacco Inc. (Tokyo, Japan). The construction of pSB11 from pSB21 and the construction of pSB21 from starting vectors is described by Komari et al. (1996, Plant J. 10:165-174). The T-DNA of pPHP8904 was integrated into the superbinary plasmid pSB1 (Saito et al., EP 672 752 A1) by homologous recombination between the two plasmids (Fig. 1, pSB1 x pPHP8904). The plasmid pSB1 was also obtained from Japan Tobacco Inc. *E. coli* strain HB101 containing pPHP8904 was mated with *Agrobacterium* strain LBA4404 harboring pSB1 to create the cointegrate plasmid in *Agrobacterium*, designated as LBA4404(pPHP10525) as shown in Fig. 1c, using the method of Ditta et al., (*Proc. Natl. Acad. Sci. USA* 77:7347-7351, 1980). LBA4404(pPHP10525)

was selected based on resistance of transformed *Agrobacterium* to spectinomycin and verified as a recombinant by a SalI restriction digest of the plasmid.

Example 2
Transformation of A188 using the protocol of Ishida et al.

Transformation of A188 was performed according to the protocol of Ishida et al. except that 1.5 mg/l bialaphos was used during the first two weeks of the selection step while 3 mg/l bialaphos was used for the remainder of the selection period (Ishida et al. used 5 mg/l phosphinothricin (PPT) during the first two weeks of selection and 10 mg/l PPT during the remainder of the selection period). Use of bialaphos compared to PPT resulted in a lower initial frequency of recovery of callus lines growing on herbicide and plants regenerated on herbicide, but the final frequency of confirmed transformed plants, as determined by expression of the introduced GUS gene, is similar using the two herbicides. This difference can be attributed to the tighter selection achieved using bialaphos rather than PPT. PPT leads to a higher number of escapes, i.e., plants regenerated from herbicide-selected callus that are not actually transformed (Dennehey et al. *Plant Cell, Tissue and Organ Culture* 36:1-7, 1994). Therefore, in all subsequent experiments, bialaphos was used as the selective agent. The same source of A188 seed was used as that of Ishida et at. The *Agrobacterium* strain used was LBA4404(pPHP10525), which was identical to the strain used in Ishida et al. except 2X35S-bar replaced 35S-bar and Ubi-intron-GUS replaced 35S-intron-GUS (Details are provided in Example 1). The results of the transformation are summarized in Table 3. The proportion of embryos which produced herbicide-resistant callus and the proportion of embryos which produced regenerated plants was higher using the modified method of Ishida et al. (compare Tables 1 and 3), although the final stable transformation frequencies are similar (compare B/A in Table 1 and Table 3). This result can be attributed to the tendency for tighter selection and fewer escapes when bialaphos is used rather than PPT. GUS+ plants refer to those plants which showed positive expression of

the GUS gene, as determined by the standard X-gluc assay (McCabe, D.E., 1988, *Biotechnol.* 6:923-926).

Table 3. Transformation of A188 using the protocol of Ishida et al.

	Number of Immature Embryos				
Experiment No.	Inoculated (A)	Callus growing on herbicide	Plants regenerated on herbicide	GUS+ plants (B)	Frequency (B/A, %)
1	150	28	20	16	10.7%
2	180	21	19	16	8.9%
3	50	9	9	9	18.0%
4	157	28	18	16	10.2%

As demonstrated in Table 3, the range of transformation frequencies was within the range reported by Ishida et al.

Example 3
Transformation of Hi-II using the protocol of Ishida et al.

The protocols of Ishida et al. (*supra*) were used in this Example. As in Example 2, bialaphos was substituted for PPT. The *Agrobacterium* strain LBA4404(pPHP10525) described in Example 1 and used in Example 2 was also used in this Example. "Hi-II" was derived by reciprocal crosses between plants of Hi-II Parent A and plants of Hi-II Parent B (both parents available from the Maize Genetic Cooperation Stock Center, Univ. of Illinois at Champaign/Urbana, Illinois). Seeds recovered from the crosses were termed Hi-II seeds. Hi-II seeds were planted either in a greenhouse or a field. The resulting Hi-II plants were either self-

pollinated or cross-pollinated with sister plants. Immature embryos were isolated from ears harvested between about 9-13 days after pollination. The embryos used for these experiments were generally in the 1.0-1.2 mm size range. GUS+ events were determined at the callus stage or regenerated plant stage. The results using the Ishida et al. protocol (see summary of protocol in Table 2) are summarized in Table 4.

Table 4. Results on transformation of Hi-II using the protocol of Ishida et al.

	Number of Immature Embryos		
Experiment No.	Inoculated	Produced GUS+ events	Frequency
1	165	4	2.4%
2	30	1	3.3%
3	205	6	2.9%
4	87	1	1.1%
5	177	8	4.5%
6	56	4	7.1%
7	80	1	1.3%
8	120	1	0.8%
9	115	5	4.3%
10	58	4	6.9%

The results indicated that the transformation frequencies for Hi-II (a cross between A188 and B73) were within the range reported by Ishida et al. for crosses of A188 with a variety of other inbreds and provided a basis for comparing other transformation protocols.

Example 4
Transformation of Hi-II using PHI protocols

Preparation of *Agrobacterium* suspension: *Agrobacterium* was streaked out from a -80° frozen aliquot onto a plate containing PHI-L medium and cultured at 28°C in the dark for 3 days. PHI-L media comprised 25 ml/l Stock Solution A, 25 ml/l Stock Solution B, 450.9 ml/l Stock Solution C and spectinomycin (Sigma Chemicals) added to a concentration of 50 mg/l in sterile ddH_2O (stock solution A: K_2HPO_4 60.0 g/l, NaH_2PO_4 20.0 g/l, adjust pH to 7.0 w/KOH and autoclave; stock solution B: NH_4Cl 20.0 g/l, $MgSO_4.7H_2O$ 6.0 g/l, KCl 3.0 g/l, $CaCl_2$ 0.20 g/l, $FeSO_4.7H_2O$ 50.0 mg/l, autoclave; stock solution C: glucose 5.56g/l, agar 16.67 g/l (#A-7049, Sigma Chemicals, St. Louis, MO) and autoclave).

The plate can be stored at 4°C and used usually for about 1 month. A single colony was picked from the master plate and streaked onto a plate containing PHI-M medium [yeast extract (Difco) 5.0 g/l; peptone (Difco)10.0 g/l; NaCl 5.0 g/l; agar (Difco) 15.0 g/l; pH 6.8, containing 50 mg/L spectinomycin] and incubated at 28°C in the dark for 2 days.

Five ml of either PHI-A, [CHU(N6) basal salts (Sigma C-1416) 4.0 g/l, Eriksson's vitamin mix (1000X, Sigma-1511) 1.0 ml/l; thiamine.HCl 0.5 mg/l (Sigma); 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma) 1.5 mg/l; L-proline (Sigma) 0.69 g/l; sucrose (Mallinckrodt) 68.5 g/l; glucose (Mallinckrodt) 36.0 g/l; pH 5.2] for the PHI basic medium system, or PHI-I [MS salts (GIBCO BRL) 4.3 g/l; nicotinic acid (Sigma) 0.5 mg/l; pyridoxine.HCl (Sigma) 0.5 mg/l; thiamine.HCl 1.0 mg/l; myo-inositol (Sigma) 0.10 g/l; vitamin assay casamino acids (Difco Lab) 1.0 g/l; 2, 4-D 1.5 mg/l; sucrose 68.50 g/l; glucose 36.0 g/l; adjust pH to 5.2 w/KOH and filter-sterilize] for the PHI combined medium system and 5 μl of 100 mM (3'-5'-Dimethoxy-4'-hydroxyacetophenone, Aldrich chemicals) were added to a 14 ml Falcon tube in a hood. About 3 full loops (5 mm loop size) *Agrobacterium* was collected from the plate and suspended in the tube, then the tube was vortexed to make an even suspension. One ml of the suspension was

transferred to a spectrophotometer tube and the OD of the suspension was adjusted to 0.72 at 550 nm by adding either more *Agrobacterium* or more of the same suspension medium. The *Agrobacterium* concentration was approximately 1 x 10^9 cfu/ml. The final *Agrobacterium* suspension was aliquoted into 2 ml microcentrifuge tubes, each containing 1 ml of the suspension. The suspensions were then used as soon as possible.

Embryo isolation, infection and co-cultivation:

About 2 ml of the same medium (here PHI-A or PHI-I) used for the *Agrobacterium* suspension were added into a 2 ml microcentrifuge tube. Immature embryos were isolated from a sterilized ear with a sterile spatula (Baxter Scientific Products S1565) and dropped directly into the medium in the tube. A total of about 100 embryos were placed in the tube. The optimal size of the embryos was about 1.0-1.2 mm. The cap was then closed on the tube and the tube was vortexed with a Vortex Mixer (Baxter Scientific Products S8223-1) for 5 sec. at maximum speed. The medium was removed and 2 ml of fresh medium were added and the vortexing repeated. All of the medium was drawn off and 1 ml of *Agrobacterium* suspension was added to the embryos and the tube vortexed for 30 sec. The tube was allowed to stand for 5 min. in the hood. The suspension of *Agrobacterium* and embryos was poured into a Petri plate containing either PHI-B medium [CHU(N6) basal salts (Sigma C-1416) 4.0 g/l; Eriksson's vitamin mix (1000X, Sigma-1511) 1.0 ml/l; thiamine.HCl 0.5 mg/l; 2.4-D 1.5 mg/l; L-proline 0.69 g/l; silver nitrate 0.85 mg/l; gelrite (Sigma) 3.0 g/l; sucrose 30.0 g/l; acetosyringone 100 μM; pH 5.8], for the PHI basic medium system, or PHI-J medium [MS Salts 4.3 g/l; nicotinic acid 0.50 mg/l; pyridoxine HCl 0.50 mg/l; thiamine.HCl 1.0 mg/l; myo-inositol 100.0 mg/l; 2, 4-D 1.5 mg/l; sucrose 20.0 g/l; glucose 10.0 g/l; L-proline 0.70 g/l; MES (Sigma) 0.50 g/l; 8.0 g/l agar (Sigma A-7049, purified) and 100 μM acetosyringone with a final pH of 5.8 for the PHI combined medium system. Any embryos left in the tube were transferred to the plate using a sterile spatula. The *Agrobacterium* suspension

was drawn off and the embryos placed axis side down on the media. The plate was sealed with Parafilm tape or Pylon Vegetative Combine Tape (product named "E.G.CUT" and is available in 18 mm x 50 m sections; Kyowa Ltd., Japan) and incubated in the dark at 23-25°C for about 3 days of co-cultivation.

Resting, selection and regeneration steps:

For the resting step, all of the embryos were transferred to a new plate containing PHI-C medium [CHU(N6) basal salts (Sigma C-1416) 4.0 g/l; Eriksson's vitamin mix (1000X Sigma-1511) 1.0 ml/l; thiamine.HCl 0.5 mg/l; 2.4-D 1.5 mg/l; L-proline 0.69 g/l; sucrose 30.0 g/l; MES buffer (Sigma) 0.5 g/l; agar (Sigma A-7049, purified) 8.0 g/l; silver nitrate 0.85 mg/l; carbenicillin 100 mg/l; pH 5.8]. The plate was sealed with Parafilm or Pylon tape and incubated in the dark at 28°C for 3-5 days.

For selection, all of the embryos were then transferred from the PHI-C medium to new plates containing PHI-D medium, as a selection medium, [CHU(N6) basal salts (SIGMA C-1416) 4.0 g/l; Eriksson's vitamin mix (1000X, Sigma-1511) 1.0 ml/l; thiamine.HCl 0.5 mg/l; 2.4-D 1.5 mg/l; L-proline 0.69 g/l; sucrose 30.0 g/l; MES buffer 0.5 g/l; agar (Sigma A-7049, purified) 8.0 g/l; silver nitrate 0.85 mg/l; carbenicillin (ICN, Costa Mesa, CA) 100 mg/l; bialaphos (Meiji Seika K.K., Tokyo, Japan) 1.5 mg/l for the first two weeks followed by 3 mg/l for the remainder of the time.; pH 5.8] putting about 20 embryos onto each plate. The plates were sealed as described above and incubated in the dark at 28°C for the first two weeks of selection. The embryos were transferred to fresh selection medium at two week intervals. The tissue was subcultured by transferring to fresh selection medium for a total of about 2 months. The herbicide-resistant calli were then "bulked up" by growing on the same medium for another two weeks until the diameter of the calli was about 1.5-2 cm.

For regeneration, the calli were then cultured on PHI-E medium [MS salts 4.3 g/l; myo-inositol 0.1 g/l; nicotinic acid 0.5 mg/l, thiamine.HCl 0.1 mg/l, Pyridoxine.HCl 0.5 mg/l, Glycine 2.0 mg/l, Zeatin 0.5 mg/l, sucrose 60.0 g/l, Agar

(Sigma, A-7049) 8.0 g/l, Indoleacetic acid (IAA, Sigma) 1.0 mg/l, Abscisic acid (ABA, Sigma) 0.1 μM, Bialaphos 3 mg/l, carbenicillin 100 mg/l adjusted to pH 5.6] in the dark at 28°C for 1-3 weeks to allow somatic embryos to mature. The calli were then cultured on PHI-F medium (MS salts 4.3 g/l; myo-inositol 0.1 g/l; Thiamine.HCl 0.1 mg/l, Pyridoxine.HCl 0.5 mg/l, Glycine 2.0 mg/l, nicotinic acid 0.5 mg/l; sucrose 40.0 g/l; gelrite 1.5 g/l; pH 5.6] at 25°C under a daylight schedule of 16 hrs. light (270 uE $m^{-2}sec^{-1}$) and 8 hrs. dark until shoots and roots developed. Each small plantlet was then transferred to a 25x150 mm tube containing PHI-F medium and grown under the same conditions for approximately another week. The plants were transplanted to pots with soil mixture in a greenhouse. GUS+ events were determined at the callus stage or regenerated plant stage. The results are summarized in Table 5.

For Hi-II a preferred optimized protocol was 0.5 x 10^9 cfu/ml *Agrobacterium* (Table 6), a 3-5 day resting step (Example 5), and no $AgNO_3$ in the infection medium (PHI-A medium). The examples of this invention provide a variety of experiments that similarly teach those of ordinary skill in the art to optimize transformation frequencies for other maize lines.

Table 5. **Results on transformation of Hi-II using the PHI protocols**

	Number of Immature Embryos			
Medium	Experiment No.	Inoculated	Produced GUS+events	Frequency
PHI basic	1	195	64	32.8%
	2	97	37	38.1%
	3	65	30	46.2%
	4	103	52	50.5%
PHI combined	1	65	5	7.7%
	2	51	4	7.8%
	3	98	22	22.4%
	4	71	8	11.3%

The results indicated that the PHI-combined medium system gave higher transformation frequencies than Ishida's protocol. On average, 13.7% stable transformants were generated with the PHI combined medium system, while 3.2% stable transformants were produced using the protocol of Ishida et al. Thus, the PHI combined medium system was about 4.3 times better than the Ishida et al. Protocol for Hi-II transformation. The transformation frequency was also very high with the PHI basic medium system producing transformation frequencies that were about 12.4 times better than the Ishida et al. protocol.

Experiments were also designed to develop a method for optimizing the concentration of *Agrobacterium* to be used for maize transformation. The same procedures were used as described for the experiments summarized in Table 5, with the following modifications in *Agrobacterium* preparation methods. For final concentrations of *Agrobacterium* other than 1 x 10^9 cfu/ml, a concentrated *Agrobacterium* suspension can initially be made. The concentration of the *Agrobacterium* suspensions is determined by measuring the OD value at 550 nm of a dilution or serial dilution that gives a reading of approximately OD_{550}=0.72. The

concentration of the *Agrobacterium* suspensions are then adjusted to the desired concentration for use in transformation experiments. In the experiments summarized in Table 6, the working suspension of *Agrobacterium* used were 10 x 10^9, 2 x 10^9, 1 x 10^9, 0.5 x 10^9, 0.1 x 10^9 (all in cfu/ml) and GUS+ events were determined at the callus stage or regenerated plant stage. The results are shown in Table 6.

The results of Table 6 indicated that for both medium systems, transformation frequency was affected by *Agrobacterium* concentration. The highest transformation frequency was obtained using 0.5 x 10^9 cfu/ml *Agrobacterium* at the infection step for both medium systems for Hi-II embryos. The transformation frequency with 0.5 x 10^9 cfu/ml and PHI-basic medium system as provided in Table 6 is not as high as the transformation frequency in Table 5, because an old version of PHI-A (containing AgNo3, 2.0 mg/l 2,4-D, at pH 5.8) was used in the experiments with results provided in Table 6. It is likely that the transformation frequencies for the PHI-basic medium system could be improved by removing $AgNO_3$ in the infection medium and using 1.5 mg/l 2-4,D with the infection medium at pH 5.2.

Table 6. Results of experiments to optimize *Agrobacterium* concentrations

		Agrobacterium Concentration (cfu/ml)														
		1 x 10^{10}			2 x 10^{9}			1 x 10^{9}			0.5 x 10^{9}			0.1 x 10^{9}		
Med.	Exp. No.	Embryos			Embryos			Embryos			Embryos			Embryos		
		Inocu- lated	GUS+ events	%	Inocu- lated	GUS+ events	%	Inocu- lated	GUS+ events	%	Inocu- lated	GUS+ events	%	Inocu -lated	GUS+ events	%
PHI comb.	1	138	1	0.7	64	2	3.1	66	2	3.0	51	4	7.8		ND*	
	2	86	1	1.2	58	1	1.7	55	4	7.3	65	5	7.7			
	3	85	6	7.1	58	3	5.2	50	3	6.0	98	22	22.4			
	4	192	13	6.8	67	4	6.0	71	8	11.3						
PHI basic	1		ND*			ND*		88	3	3.4	127	27	21.3	150	16	10.7
	2							134	9	6.7	86	15	17.4	242	17	7.0
	3							110	9	8.2	280	21	7.5	97	5	5.2
	4							85	2	2.4	196	15	7.7			

* Experiment not performed

Example 5
Hi-II Transformation including a Resting Step

Hi-II embryos were isolated and transformed as provided in Examples 3 and 4. The experiments compared PHI basic and PHI combined media systems with or without a four day resting step and contrasted the data to matched experiments using the transformation protocol of Ishida et al. as provided in Examples 3. As described in Example 2, the Ishida et al. protocol was identical to that described by Ishida et al. (*supra*) except that bialaphos replaced PPT.

The PHI basic medium system was identical to that described in Example 4 except that PHI-A medium additionally included 0.85 mg/l $AgNO_3$, another 0.5 mg/l 2,4-D at pH 5.8 for the infection step. PHI-D medium was prepared as described in Example 4 but without proline or MES buffer. An additional 0.5 mg/l 2,4-D was added in the selection step. In addition, 3 g/l gelrite was used to replace agar in the selection medium. The PHI combined medium system was also identical to that of Example 4 except that proline and MES buffer were not added to the PHI-D medium and 2,4-D was used at a concentration of 2.0 mg/l and 3 g/l of Gelrite was used to replace the agar in the selection step. Results are provided in Table 7 below.

Results indicated that the addition of a 4-day resting step can increase the transformation frequency about 2.7 times as compared with the Ishida et al. method. The 4-day resting step increased the transformation frequency in the PHI combined medium system about 2.3 times. However, unexpectedly, the combination of the PHI basic protocol combined with a resting step resulted in an increase in the transformation frequency of about 15 times the frequency observed without a resting step. The combination of the protocols of this invention with a resting for Hi-II provided a significant improvement in transformation frequency.

Table 7. Hi-II Transformation With or Without Resting Step

		Without resting step			With 4-day resting		
Transformation Protocol	Experiment No.	Inoculated Embryos	Embryos producing GUS+ events	Frequency	Inoculated Embryos	Embryos producing GUS+ events	Frequency
Ishida et al.	1	165	4	2.4%	177	11	6.2%
	2	30	1	3.3%	56	5	8.9%
	Total	195	5	2.6%	233	16	6.9%
PHI basic	1	154	1	0.6%	50	8	16.0%
	2	108	1	0.9%	47	3	6.4%
	3				45	10	22.2%
	4				38	1	2.6%
	Total	262	2	0.8%	180	22	12.2%
PHI combined	1	170	14	8.2%	66	2	3.0%
	2	64	0	0.0%	55	4	7.3%
	3	230	0	0.0%	50	3	6.0%
	4				71	8	11.3%
	Total	464	14	3.0%	242	17	7.0%

Example 6
Transformation of A188 x inbred crosses using the PHI protocols

F_1 immature embryos were isolated from crosses of A188 to other inbreds and were subjected to transformation by *Agrobacterium*. The protocols used were the same as in Example 4, with the following modifications. The *Agrobacterium* suspension was prepared with either the N6 salt containing medium, PHI-G [100 ml/l of a 10X solution of N6 macronutrients (463.0 mg/l $(NH_4)_2SO_4$, 400.0 mg/l KH_2PO_4, 125.33 mg/l $CaCl_2$, 90.37 mg/l $MgSO_4$ and 2,830.0 mg/l KNO_3), 2.44 mg/l Boric acid, 37.1 mg/l Na_2-EDTA.$2H_2O$, 27.88 mg/l FeSO4.$7H_2O$, 7.33 mg/l $MnSO_4.H_2O$, 0.77 mg/l KI, 0.6 mg/l $ZnSO_4.7H_2O$, 0.15 mg/l $Na_2MoO_2.2H_2O$, 1.68 g/l KNO_3, 0.8 mg/l glycine, 3.2 mg/l nicotinic acid, 3.2 mg/l Pyridoxine.HCl, 3.4 mg/l Thiamine.HCl, 0.6 g/l Myo-inositol, 0.8 mg/l 2,4-D, 1.2 mg/l Dicamba (Sigma), 1.98 g/l L-proline, 0.3 g/l casein hydrolysate, 68.5 g/l sucrose and 36.0 g/l glucose, pH 5.2] or the MS salt-containing medium, PHI-I (*supra*) for the infection step. The co-cultivation medium was PHI-J (*supra*) and the co-cultivation time was about 3 to about 7 days. For PHJ90 x A188, PHI-C medium (*supra*) was used in a 3 day resting step and PHI-D medium (*supra*) was used for selection. For PHN46 x A188 and PHPP8 x A188 transformations, no resting step was used, the co-cultivation time was about 5-7 days, and PHI-H medium [100 ml/l of a 10X solution of N6 macronutrients (463.0 mg/l $(NH_4)_2SO_4$, 400.0 mg/l KH_2PO_4, 125.33 mg/l $CaCl_2$, 90.37 mg/l $MgSO_4$ and 2,830.0 mg/l KNO_3), 2.44 mg/l Boric acid, 37.1 mg/l Na_2-EDTA.$2H_2O$, 27.88 mg/l FeSO4.$7H_2O$, 7.33 mg/l $MnSO_4.H_2O$, 0.77 mg/l KI, 0.6 mg/l $ZnSO_4.7H_2O$, 0.15 mg/l $Na_2MoO_2.2H_2O$, 1.68 g/l KNO_3, 0.8 mg/l glycine, 3.2 mg/l nicotinic acid, 3.2 mg/l Pyridoxine.HCl, 3.4 mg/l Thiamine.HCl, 0.6 g/l Myo-inositol, 1.0 mg/l 2,4-D, 1.0 mg/l Dicamba, 0.3 g/l casein hydrolysate, 20.0 g/l Sucrose, 0.6 g/l glucose, 0.5 g/l MES buffer, 1 mg/l AgNO3, 5 mg/l bialaphos, 100 mg/l carbenicillin and 8.0 g/l Agar (Sigma A-7049, purified); pH 5.8] was used for selection. GUS+ events were determined at the

callus stage or could be determined at the regenerated plant stage. The results are summarized in Table 8.

These results indicate that the methods of this invention produced an improved transformation frequency for crosses of A188 to the inbreds with transformation frequencies ranging from about 6.9% to 50.5 % (citing data including the Hi-II studies from Table 5). These transformation frequencies were significantly higher than the frequencies reported by Ishida et al. for A188 x inbred crosses which ranged from about 0.4 to 5.3%.

Table 8. Results of transformation of A188 x inbred crosses using the PHI protocols

		Number of Immature Embryos		
Variety	Experiment No.	Inoculated	Produced GUS+events	Frequency
PHJ90 x A188	1	151	72	47.7%
	2	85	36	42.4%
PHN46 x A188	1	112	46	41.1%
	2	80	37	46.3%
	3	114	47	41.2%
	4	51	8	15.7%
PHPP8 x A188	1	160	11	6.9%
	2	109	8	7.3%
	3	141	27	19.1%

Example 7
Transformation of elite inbreds using the PHI protocols

For transformation of elite inbred lines, the protocols described in Example 4 were used, with the following modifications. For PHJ90, the *Agrobacterium* suspension was prepared with either PHI-A (PHI basic medium) or PHI-I (PHI combined medium), the co-cultivation medium was either PHI-B (PHI basic medium) or PHI-J (PHI combined medium), the co-cultivation time was about 3 to 5 days, PHI-C medium was used for a resting step of about 3-5 days, and PHI-D medium was used for selection. For PHN46, the *Agrobacterium* suspension was prepared with either PHI-G or PHI-I (both PHI combined media), the co-cultivation media was PHI-J, the co-cultivation time was about 7 days, no resting step was used, and PHI-H medium was used for selection. For PHP~~28~~38, the *Agrobacterium* suspension was prepared with PHI-I (PHI combined media), the co-cultivation medium was PHI-J, the co-cultivation time was about 3 days, PHI-H without bialaphos was used for a resting step of about 4 days and PHI-H medium was used for selection. Formulations for the media referenced in this example are detailed in either Example 4-6. GUS+ events were determined at the regenerated plant stage. The results are summarized in Table 9. Using the protocol of Ishida, et al., no stable transformants were recovered from 594 embryos in 5 separate experiments for PHJ90, 644 embryos in 4 separate experiments for PHN46, and 263 embryos in 4 separate experiments for PHP~~28~~38.

Table 9. Results on transformation of elite inbreds using the PHI protocols

		Number of Immature Embryos		
Variety	Experiment No.	Inoculated	Produced GUS+plants	Frequency
PHJ90	1	85	1	1.2%
	2	58	1	1.7%
	3	164	2	1.2%
	4	60	3	5.0%
PHN46	1	44	3	6.8%
	2	49	4	8.2%
	3	114	8	7.0%
	4	123	11	8.9%
PHP~~28~~ 38	1	89	2	2.2%
	2	104	1	1.0%
	3	116	1	0.9%

The results demonstrated that both the PHI basic medium and the PHI combined medium which use N6 salts for various steps in the protocols and carbenicillin led to recovery of stably transformed calli and plants (Table 9), at frequencies from ~1.0% to 9.0%. Each of these inbred lines belongs to a different heterotic group, so the methods of the present invention enable one not only to transform inbred lines across a wide range of genotypes and to transform inbred lines of commercial importance. Some inbreds (e.g., PHN46) could be transformed without a resting step, but the resting step was important for some inbreds such as PHJ90. Those skilled in the art will recognize that the transformation protocols of this invention can be performed in duplicate with or without the addition of a resting step without undue experimentation. These results are significant because they

demonstrate that the methods of this invention can be used to transform a variety of elite lines.

The effect of various combinations of co-cultivation periods and resting periods were also reviewed for these elite inbreds and the results of these experiments are summarized below:

Table 10: Combinations of Co-cultivation and Resting Time for Elite Inbreds

Inbred	Length of Co-cultivation (days)	Length of Resting (days)	Transformation Frequency Range (%)
PHN46	3	0	0.6-3.4
	5	0	2
	7	0	0.6-8.9
	10	0	1.4-3.4
PHP~~28~~38	3	4	0.9-2.2
	7	0	2.2-14.5
PHJ90	3	0	0
	3	3	0.6-1.2
	3	4	1.2-1.7

These data indicates that the resting step is important for some inbreds using a 3 day co-cultivation period but that longer co-cultivation periods may compensate for the absence of a resting step since the resting step, like the co-cultivation step, provides a period of time for the embryo to be cultured in the absence of a selective agent. Those of ordinary skill in the art can readily test combinations of co-cultivation and resting times to optimize or improve the transformation frequency of other inbreds without undue experimentation. Moreover, since these inbreds are representative of three different heterotic groups,

these methods demonstrate that they can be extended to other heterotic groups or to additional inbreds within the heterotic groups represented here.

It will be appreciated by those skilled in the art that while the invention has been described above in connection with particular embodiments and examples, the invention is not necessarily so limited and that numerous other embodiments, examples, uses, modifications and departures from the embodiments, examples and uses may be made without departing from the inventive scope of this application.